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Determination of Microbial Recovery for some Pharmaceutical Raw Materials and Passive Monitoring at Production Area and Microbiology Laboratory at Egyptian Pharmaceutical Company

Mohamed N. Mohamed[1]*, Mohammed S. Azab[2], Amr Fouda[2], Saad El-Din Hassan[2], Salah Abdelbary[2]

[1]Sector of Research and Development, Department of Microbiology, Nile Company for Pharmaceutical and Chemical Industry, Cairo, Egypt. [2] Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Nasr City, Cairo, Egypt.

***Corresponding author:** Mohamed N. Mohamed, [1]Sector of Research and Development, Department of Microbiology, Nile Company for Pharmaceutical and Chemical Industry, Cairo, Egypt. Tel. (+2)01027577872*
Email address: Mohamednabil1986@yahoo.com

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ABSTRACT

Objective: This study aims to evaluate the validity of pharmaceutical raw materials obtained from an Egyptian pharmaceutical factory to ensure the accuracy of the examination findings before they are used in the manufacturing process. On the other hand, it also aims to determine the environmental microbial count at the microbiology laboratory and production areas to prevent microbial contamination during production and during microbiological analysis. **Methods:** Assessment of microbiological quality control according to pharmacopeia as plate method by using TSB media with a concentration of 0.1% tween 80, for passive monitoring using settled plate (TSA) media. **Results:** the percentage of microbial recovery for lactose monohydrate was 89%, 85%, 94%, 92%, 91%, and 91%, while Croscarmellose recorded 81%, 85%, 85%, 85%, and 88%, Mannitol recorded 86%, 85%, 91%, 83%, 86%, and 88%, Magnesium stearate recorded 91%, 85%, 94%, 89%, and 91%, and finally, Talc Powder recorded 94%, 85%, 94%, 91%, 87% and 85% for *Pseudomonas aeruginosa*, *Salmonella enterica*, *Staphylococcus aureus*, *Candida albicans*, *Bacillus subtilis* and *Escherichia coli* respectively for each pharmaceutical raw material. Methods of analysis recorded under acceptance criteria in the selected pharmaceutical factory according to pharmacopeia. On the other hand, the microbial quality of air for the production area selected by an Egyptian pharmaceutical company was under acceptance limits. **Conclusion:** All quality control methods and assessments of pharmaceutical raw materials, laboratories, and production areas must be performed to measure and control microbial contamination in the pharmaceutical industry and then produce high-quality pharmaceutical products free of microbial contamination, and methods of analysis must be recorded under acceptance criteria in the selected pharmaceutical factory according to pharmacopeia. On the other hand, the microbial quality of the air for the production area and microbiology laboratory that was selected by an Egyptian pharmaceutical company was under accepted limits. All quality control methods and assessments of pharmaceutical raw materials, laboratories, and production areas must be performed to measure and control microbial contamination in the pharmaceutical industry and then produce high-quality pharmaceutical products free of microbial contamination.

Keywords: *Microorganisms; Contamination; Quality control; Pharmaceutical industry and recovery.*

INTRODUCTION

In the pharmaceutical industry, the presence of microbial contamination on raw materials during pharmaceutical preparation causes spoilage of drugs and effects on human health[1]. Therefore, microbiological quality is a critical attribute of the raw materials used in pharmaceutical manufacturing that influences the level of bioburden of the finished product. If the preliminary treatment for the microbiological analysis of the sample is not appropriate for the recovery of the actual microbial content, incorrect microbial presence of raw material count calculation affects the quality and safety of the final product [2] . Important information on possible causes of contamination and the spread of microbial species in pharmaceutical products is provided by detecting microbial contaminants in product recalls and environmental samples. Review of FDA product recalls data from 1998 to September 2006 for 134 non-sterile pharmaceutical products. From 1998 to September 2006, it was shown that 48% of recalls were due to *Burkholderia cepacia*, *Pseudomonas* spp., or *Ralstoniapicketti* contamination, while 23% of recalls were due to yeast and mold contamination. Gram-negative bacteria represented 60% of recalls, while 4% were associated with Gram-positive bacteria. The distribution of microbial growth is constrained by environmental gradients in pharmaceutical environments [3]**.** Bacteria, yeast, and molds are the main pathogens in non-sterile pharmaceutical products and ingredients [4]. Microbial limit tests were used to evaluate raw material and non-sterile products for appropriate microbial quality for non-sterile formulation for acceptable microbial limit quality since microorganisms can cause patient diseases [5]. Microbiological consistency of the drugs should be integrated into the medicine of microbial contamination sources, the environmental conditions at the pharmaceutical production area and product attributes that promote microorganism 'growth [6]. Contamination of raw materials and during manufacture leads to products becoming contaminated during storage and use. A way to verify the methods used to check foods or drugs appropriate for bacterial control should be found when designing a microbiological test for foods or drugs. Validation of a method is the process of deciding whether a method is sufficient for its intended use [7]. Pharmaceuticals raw materials such as lactose monohydrate are used as a filler and diluents in tablets and capsules, and to a more limited extent in lyophilized products and infants, while talc powder is used in oral solid dosage formulations as a lubricant and diluents, Croscarmellose is used as a disintegrate for capsules, tablets, and granules in oral pharmaceutical formulations, Mannitol is used as a diluent in tablet formulations, moisture-sensitive active ingredients, direct compression tablet applications, Magnesium stearate is used as a lubricant in capsule and tablet manufacture at concentrations ranging from 0.25% to 5.0% w/w. is used in barrier creams [8]. Pharmacopeias offer standards, specifications, and test methods that are required to be used in the pharmaceutical industry to ensure that pharmaceuticals achieve perfect quality-control tests [9]. This current work is aimed at studying the microbial quality of the selected pharmaceutical raw materials that are commonly used in the pharmaceutical industry, as well as determining the microbial quality of the air in production areas.

MATERIAL AND METHODS

Material

Collection of non-sterile pharmaceutical raw materials

Five non-sterile raw materials that were used in the pharmaceutical industry were collected from a pharmaceutical company in Egypt as following: Lactose monohydrate, Talc powder, Croscarmellose, Mannitol not used for injection, and Magnesium stearate.

Test microorganisms that used

Test microorganisms that were used in this study: Gram-positive bacteria as *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, Gram-negative bacteria as *Pseudomonas aeruginosa* ATCC 9027, *Salmonella enterica* ATCC 14028, *Escherichia coli* ATCC 8739, and yeasts as *Candida albicans* ATCC 10231.

Methods

Total viable microbial count on non-sterile pharmaceutical raw materials

Weight an appropriate amount of pharmaceutical raw materials to be diluted with Tryptone Soya Broth (1:10). Then, 1ml from this dilution was added to a sterile petri dish filled with sterile Tryptone Soya Agar (TSA) medium for the bacterial count and Sabouraud Dextrose Agar (SDA) medium for the fungal count [10].This equation must be used to calculate the number of colony forming units (CFU):CFU/ml = a number of colonies count ×dilution factor/Amount of sample volume added to the plate [11].

Validation of pharmaceutical raw materials

Preparation of inoculum

Bacterial strains were inoculated on Casein Soya Bean Digest Agar (SCDA) then incubated for 18-24 hours at 32.5°C. *Candida albicans* inoculated on SDA at 20-25°C for 2-3 days[10]. Preparation of buffered sodium chloride peptone solution pH 7.0 was done to make test suspensions to be 1×10^8 CFU/ml**[12]**. For raw

material validation, use serial dilution to reduce the dilution to 100 CFU/ml [13], then take 0.1ml from these dilutions to be added on Tryptone Soya Broth has 0.1%tween with raw materials to a final product concentration of 10^{-1} and microbial concentration be around 10-25 CFU/ml [14].

Preparation of growth promotion of used media

The media used in this study is used to promote growth in microorganisms such as gram positive bacteria *Bacillus subtillis*, *Staphylococcus aureus*, and gram negative bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica*, fungi *Candida albicans*, and which can affect the outcome of the test count results. The buffered dilution used peptone water (1g/L). Prepared all microorganisms to be less than (100 cfu/ml) for SCDA and SDA and Tryptone Soya Broth, 1ml aliquot from all microorganisms concentration inoculated at all medium, then incubated 30–35 °C for 24 hours for SCDA and tryptone soya broth, while SDA 20–250 °C for 2-3 days recovery must be in the range of 50-200% recovered [15,10].

Preparation of positive control

A positive control solution containing microorganisms will be diluted to be (10^{-1}) without raw materials. 1ml of culture organism has a concentration (10^{-2}) transferred to 9ml Tryptone Soya Broth 0.1% T (0.1ml from Tween 80 to 9ml Tryptone Soya Broth) to be 10^{-1} then 3ml from (10^{-1}) to three plates transferred to be 1ml from (10^{-1}) at each plate, then pour Soya Bean Casein Digest Agar at each plate incubate for 2-3 days, at 32.5 ± 2.5 [11].

Preparation of negative Control

One gram from raw materials was added to 9ml of (TryptoneSoya Broth 0.1% T) to achieve raw material concentration 10^{-1}, 3 ml 0f (10^{-1}) were added to petri dish, one ml to each of three plates, then poured (SCDA) media and incubate for 2-3 days, at 32.5 for bacteria, for *Candida albicans* inoculated on SDA at 20-25°C for 2-3 days[11,16].

Non-sterile raw materials suspension with microorganisms

One gram from raw materials was added to 9 ml of (TryptoneSoya Broth 0.1% Tween) media, then 1ml of microorganism concentration (10^{-2}) added to raw materials with (TryptoneSoya Broth 0.1% Tween) media, three ml of previous was added then pour one ml to each plate, then pour 15-20 ml (casein digest agar) media and incubate for 2-3 days, at 32.5 ± 2.5^{0}c for bacteria, and for *Candida albicans* inoculated on SDA at 20-25 °C for 2-3 days[10].

Control Media

After finished with the validation process of non-sterile raw materials, 20 ml medium of SCDA poured into a sterile petri dish with the same sterile tools and incubate for 2-3 days, at 32.5 ± 2.5 °C to ensure there is no contamination happen during working on validation[11, 15].

Evaluation of environmental conditions in pharmaceutical factory

Air monitoring

To identify evolving patterns in microbial counts and microflora growth in cleanrooms or regulated environments, environmental testing defines the microbiological tests conducted, information on the physical structure of the room, heating, ventilation, and air conditioning (HVAC) system efficiency was given[17].

Passive air sampling by settled plate

Sterile growth media containing Petri dishes were exposed to the specific environmental duration of time, usually about 30-60 minutes, but may be exposed to four hours before compromising the integrity of the media itself. After the plates are incubated, viable microorganisms which settle on to the media surface will develop. However, with an accurately measured volume of air, passive air sampling appears to be phased out because it does not indicate microbial contamination [18].

Data analysis

All experiments were carried out by triplicate sample. Values reported in this study were the means ± Standard Deviation and ± Standard Error using Sigma Plot software program**.**

RESULTS AND DISCUSSION

Total microbial count on non-sterile pharmaceutical raw materials

Results in **Table 1** showed that the total viable microbial count includes a bacterial and fungal count of pharmaceutical raw materials. The results recorded Zero CFU/gram on pharmaceutical raw materials, so these raw materials were suitable for the microbial limit test. Validation must be used to determine the recovery percentage and if there any inhibitory effect that can affect the routine method or intended method used to be a dependent method, from compression the result of the microbial limit test of raw materials and the standard, the results are within specification limit. Microbial limit tests were important for pharmaceutical non -sterile terms to ensure that pharmaceutical terms were free from any microbial contamination that was similar to [19] and dissimilar with [20] that found around 50% of all tested products were heavily contaminated by *Klebsiella*, *Bacillus*, and *Candida* species.

Table 1. Total microbial count on non-sterile pharmaceutical raw materials

Pharmaceutical raw materials	Microbial count CFU/g		Standard count CFU/g	
	TAMC	TYMC	TBC	TYC
Lactose Monohydrate	**<1**	**<1**	**≤100**	**≤50**
Croscarmellose sodium	**<1**	**<1**	**≤100**	**≤100**
Mannitol not intended use for parenteral	**<1**	**<1**	**≤100**	**≤100**
Magnesium stearate	**<1**	**<1**	**≤100**	**≤100**
Talc powder	**<1**	**<1**	**≤100**	**≤100**

***(CFU/g)** = colony forming unit, **(TAMC)**= total aerobic count, **(TYMC)**= total yeast mold count, **(TBC)**= total bacterial count, **TYC**= total yeast count, **(<1)** = Less than one and **(≤)** less than or equal.*

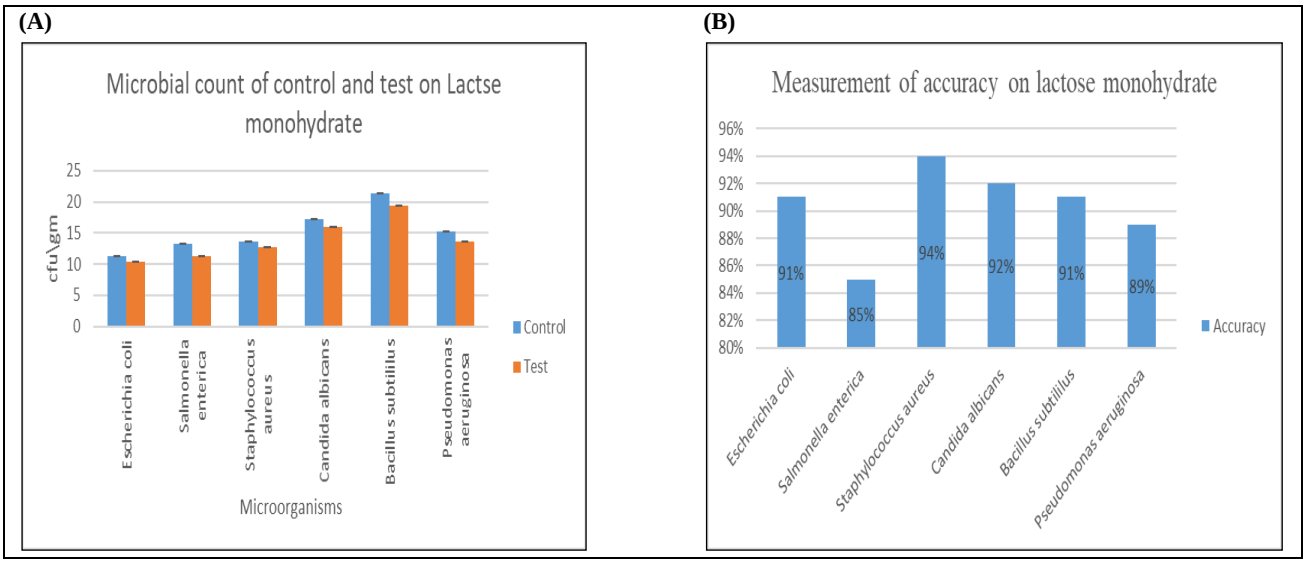

Figure 1. Evaluation of microbial quality control methods on lactose monohydrate. A: Microbial count of control and Test. **B:** recovery percentage. **Control:** microorganisms with media, **Test**: lactose monohydrate with media and microorganisms. **Recovery percentage:** microbial count in test divided by microbial count in control×100.

Validation of pharmaceutical raw materials

Evaluation of microbial quality control methods on lactose monohydrate

Results in **Figure 1** showed that the total microbial count on lactose monohydrate. Microbial count control recorded 11.3, 13.3, 13.6, 17.3, 21.3, and 15.3 and microbial count test results were 10.3, 11.3, 12.8, 16,19.3 and 13.6 on lactose monohydrate respectively on *Escherichia coli*, *Salmonella enterica*, *Staphylococcus aureus*, *Candida albicans*, *Bacillus subtilis*, *Pseudomonas aeruginosa*. Furthermore, the percentage of lactose monohydrate recovery rate at microorganisms *Escherichia coli*, *Salmonella enterica*, *Staphylococcus aureus*, *Candida albicans*, *Bacillus subtitles*, *Pseudomonas aeruginosa*, and subsequent was 89%, 85%, 94%, 92%, 91%, and 91%.All the percentage above is more than 70%. That indicates TryptoneSoy Broth with 0.1% Tween is effective in neutralization of Lactose monohydrate. Any drug that can be subjected to microbial limit test must be revalidated to ensure the method that can be selected to be intended use is similar to [20,21] .

Evaluation of microbial quality control methods on croscarmellose

Results in **Figure 2** showed that the total microbial count on croscarmellose. Results of microbial control recorded 14, 13.6, 13.6, 15.6, 15.3, and 14.3 while the microbial count on test recorded12.6,11.6, 11.6,13.3,11.3 and 13 respectively on microorganisms

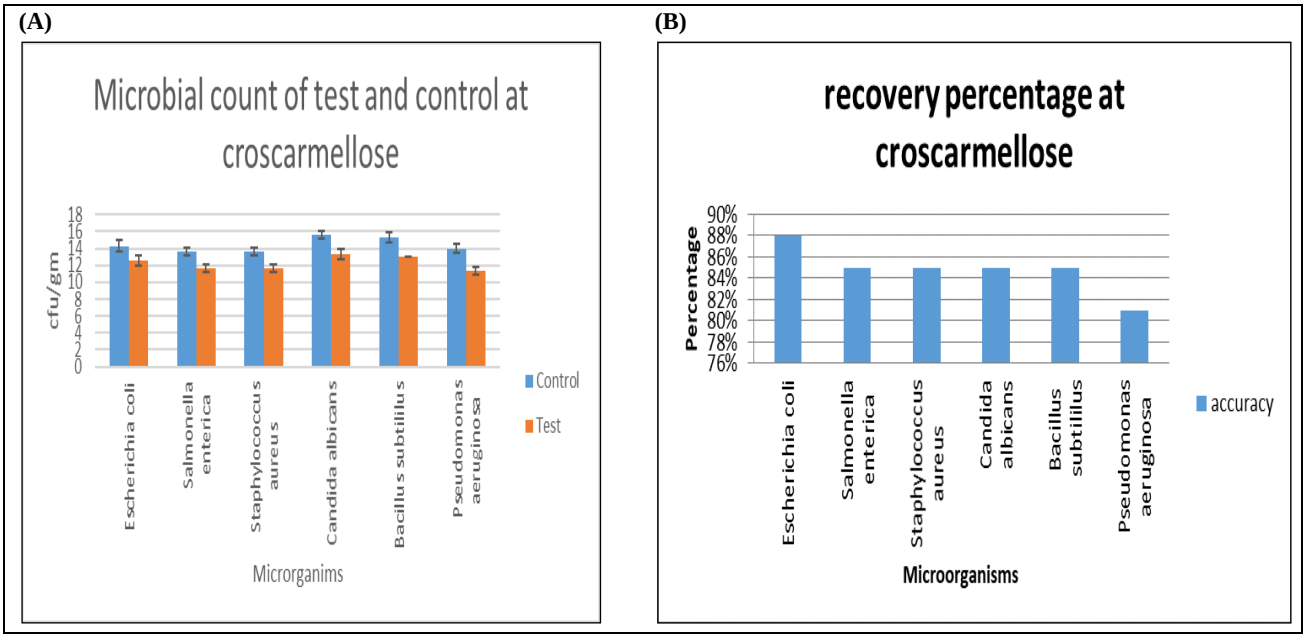

Figure 2. Evaluation of microbial quality control methods on croscarmellose. A: Microbial count of control and Test, **B:** recovery percentage, **Control:** microorganisms with media, **Test**: croscarmellose with media and microorganisms. **Recovery percentage:** microbial count in test divided by microbial count in control × 100.

Escherichia coli, *Salmonella enterica*, *Staphylococcus aureus*, *Candida albicans*, *Bacillus subtilis*, *Pseudomonas aeruginosa*. While the percentage recovery was 81%, 85%, 85%, 85%,85%, and 88%, at microorganisms *Escherichia coli*, *Salmonella enterica*, *Staphylococcus aureus*, *Candida albicans*, *Bacillus subtitles*, *Pseudomonas aeruginosa* in the following, all of the percentage above are greater than 70%, indicating that tryptone soya broth with 0.1% Tween is effective in neutralization of croscarmellose. From the result above the recovery percentage was more than 70%. The results were similar to [22].

Evaluation of microbial quality control methods on mannitol not intended use for injection

From **Figure 3**, results showed that the microbial control count and microbial test count on mannitol were not intended for use for injection. Results of control recorded 12.3, 13.6, 11.6, 17.3 ,12.3, and 11.3 while the tests were 10.6, 11.6,10.6,14.3 ,10.6 and 10, respectively on microorganisms *Escherichia coli*, *Salmonella enterica*, *Staphylococcus aureus*, *Candida albicans*, *Bacillus subtilis*, *Pseudomonas aeruginosa*. The percentage recovery of Mannitol not intended for injection was 86%, 85%, 91%, 83%, 86%, and 88% at microorganisms *Escherichia coli*, *Salmonella enterica*, *Staphylococcus aureus*, *Candida albicans*, *Bacillus subtitles*, and *Pseudomonas aeruginosa* in subsequent. The percentages above are more than 70%. That indicates tryptone soya broth with 0.1% Tween is effective in neutralization of Mannitol not intended use for injection. From the result above, the recovery percentage was more than 70%. These results were similar to[23].

Evaluation of microbial quality control methods on Magnesium stearate

Results in **Figure 4** showed that microbial control count and microbial test count on magnesium stearate control 11.6, 13.6, 13.6, 16, 18, and 15, while the test were10.6, 11.6, 11.6, 15,16 and13.6 respectively on microorganisms *Escherichia coli*, *Salmonella enterica*, *Staphylococcus aureus*, *Candida albicans*, *Bacillus subtilis*, *Pseudomonas aeruginosa* while the recovery percentage were 91%, 85%,85%, 94%,89% and 91%at microorganisms *Escherichia coli*, *Salmonella enterica*, *Staphylococcus aureus*, *Candida albicans*, *Bacillus subtitles*, *Pseudomonas aeruginosa* in subsequent. All the percentages above are more than 70% that indicates Tryptone Soya Broth with 0.1% Tween is effective in neutralization of magnesium stearate. Negative control tests were done in all tests (raw material added to media without adding microorganisms), it must be done to ensure there is no contamination during the evaluation of the accuracy of these raw materials. Any pharmaceutical raw material can be subjected to microbial limit test must be revalidated to ensure the method that can be selected to be intended use that similar to [20].

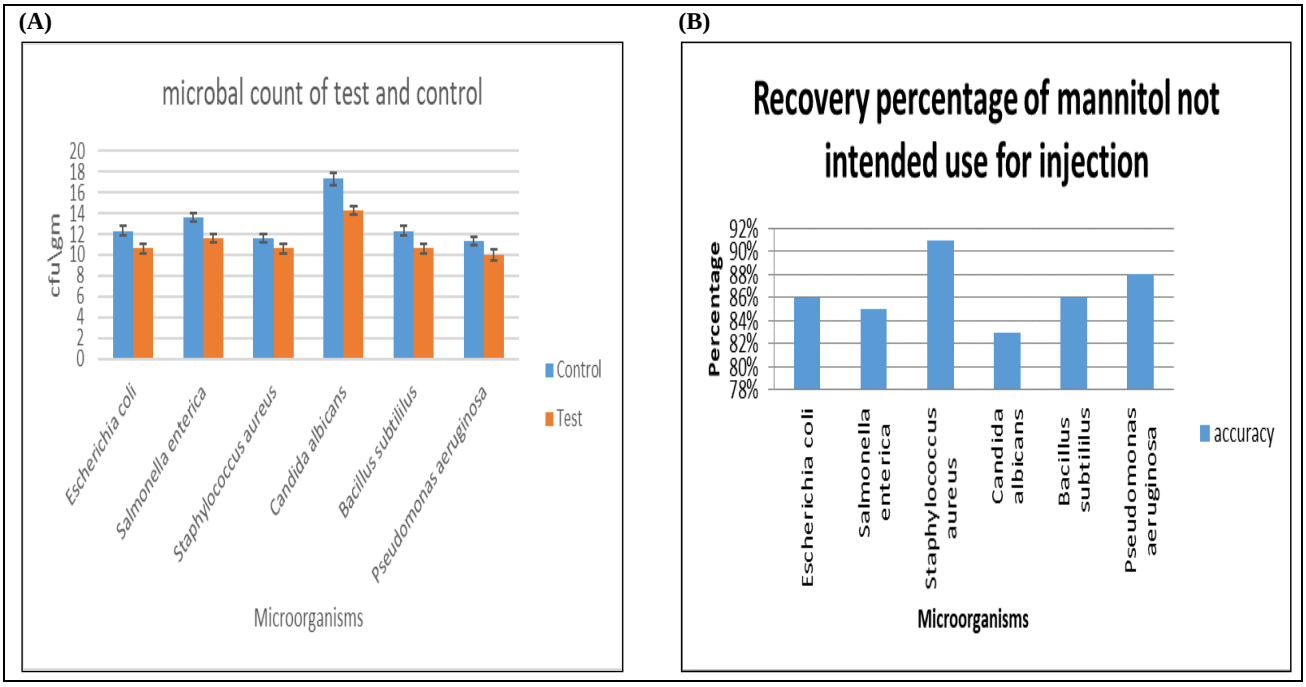

Figure 3. Evaluation of microbial quality control methods on mannitol not intended use for injection. A: Microbial count of control and Test, **B:** recovery percentage, **Control:** microorganisms with media, **Test**: mannitol not intended use for injection with media and microorganisms. **Recovery percentage:** microbial count in test divided by microbial count in control×100.

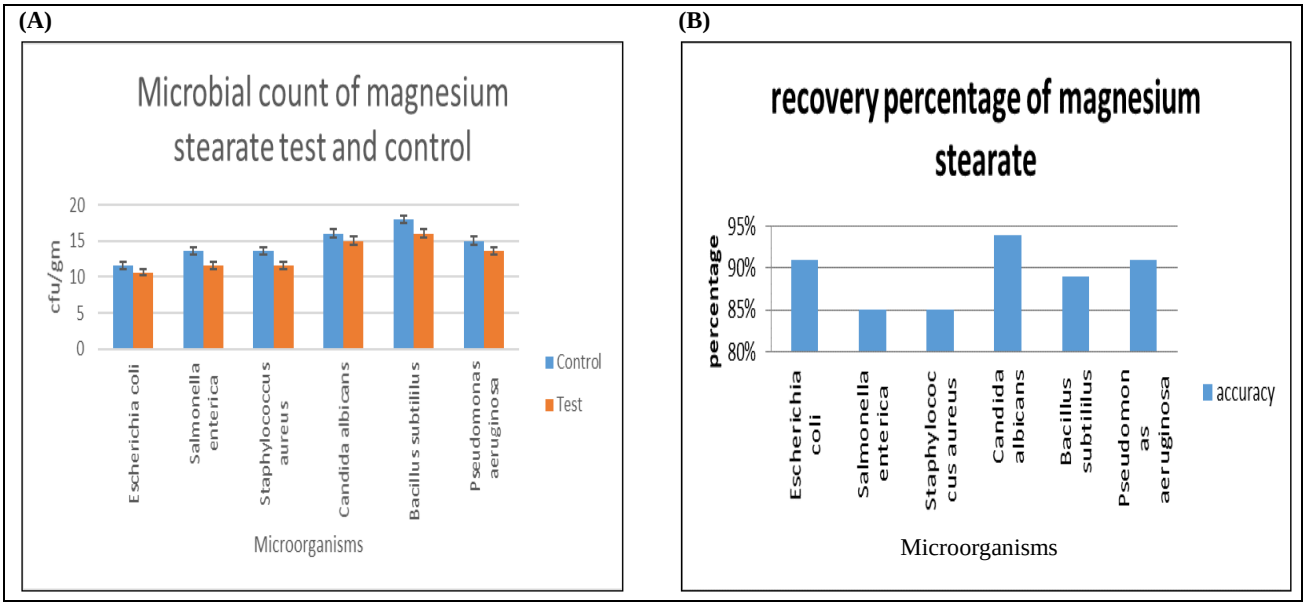

Figure 4. Evaluation of microbial quality control methods on magnesium stearate. A: Microbial count of control and Test, **B:** recovery percentage. **Control:** microorganisms with media, **Test**: Magnesium stearate with media and microorganisms. **Recovery percentage:** microbial count in test divided by microbial count in control×100.

Table 2. Microbial count at laminar airflow in microbiology laboratory

Points	TBC (TSA) CFU/Plate	TFC(SDA) CFU/Plate	Standard. Class 100 (A) (MHRA,2017&WHO, 2011)
1	Zero	Zero	Zero
2	Zero	Zero	Zero
3	Zero	Zero	Zero

Table 3. Microbial count at microbiology Laboratory as the settled plate

Points	TAMC (TSA) CFU/Plate	TFC(SDA) CFU/Plate	Standard. Class 10000 (c) CFU/Plate
1	8	1	50
2	7	Zero	50
3	6	Zero	50

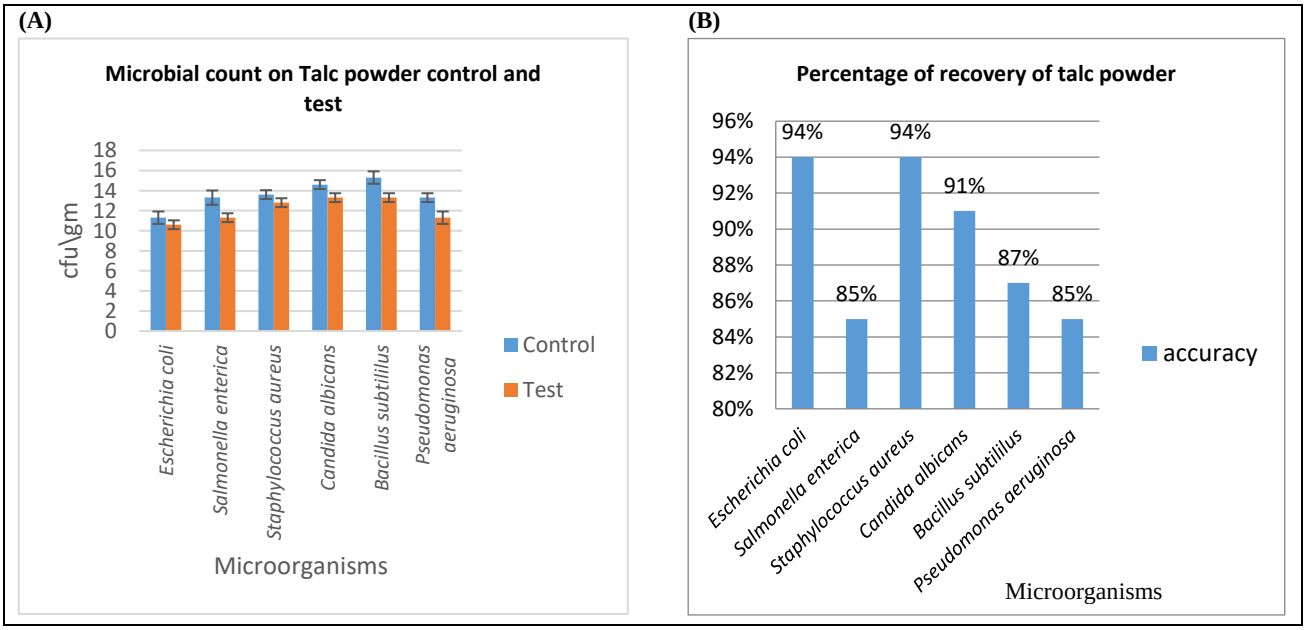

Figure 5. Evaluation of microbial quality control methods on Talc powder. A: Microbial count of control and Test, **B:** recovery percentage. **Control:** microorganisms with media, **Test**: Talc powder with media and microorganisms. **Recovery percentage:** microbial count in test divided by microbial count in control × 100.

Evaluation of microbial quality control methods on Talc powder

Results in **Figure 5** showed that the total microbial count on Talc powder. Results of microbial control recorded 11.3, 13.3, 13.6, 14.6, 15.3 and 13.3 while the microbial count on test recorded 10.6, 11.3, 12.8, 13.3, 13.3, and 11.3 respectively on microorganisms *Escherichia coli*, *Salmonella enterica*, *Staphylococcus aureus*, *Candida albicans*, *Bacillus subtilis*, *Pseudomonas aeruginosa*. While the percentage of recovery was 94%, 85%, 94%, 91%,87%, and 85%, at microorganisms *Escherichia coli*, *Salmonella enterica*, *Staphylococcus aureus*, *Candida albicans*, *Bacillus subtitles*, *Pseudomonas aeruginosa* in subsequent, all the

percentage above are more than 70%, that indicates tryptone soya broth with 0.1% Tween is effective in neutralization of croscarmellose. Negative control tests were done in all tests (raw material added to media without adding microorganisms), it must be done to ensure there is no contamination during the evaluation of the accuracy of these raw materials. Any drug that can be subjected to microbial limit test must be revalidated to ensure the method that can be selected to be intended use that similar to [20]. From the result above the recovery percentage was more than 70% these results were similar to [14].

Evaluation of environmental conditions in pharmaceutical factory

Air monitoring

Results in **Table 2** showed that the microbial count of all points under laminar airflow was Zero CFU/ml while the acceptance limit was zero CFU /ml, so the microbial count of all the points under laminar airflow under the acceptance limit was less than one CFU/Plate (Zero CFU/plate). Microbial count at microbiology laboratory and production area was under acceptance criteria these results were similar to [24,25].

Microbial count at microbiology laboratory as the settled plate

Results in **Table 3** showed that the microbial count of the three plates (TSA) for the total bacterial count (TBC) was 8 CFU/plate,7 CFU/plate,6 CFU/plate, and the three other plates(SDA) for total Fungal count (TFC) the count was 1 CFU/plate, Zero CFU/plate, Zero CFU/plate in the microbiology laboratory's test room, which was classified as class10000, and the criteria of settled plates count not more than 50 CFU/plate, All settled plates' microbial counts were under acceptance criteria. Microbial count at the microbiology laboratory and production area was under acceptance criteria. These results were similar to [17].

Microbial count at production area Class (1000) as a settled plate

Results in the **Table 4** showed that microbial count at production area as the total aerobic microbial count was 2 CFU/plate, 5 CFU/plate, 4 CFU/plate, 8 CFU/plate, 1 CFU/plate, and 3 CFU/plate, and total yeast count was Zero CFU/plate, 3 CFU/plate, 5 CFU/plate, 6 CFU/plate, 1cfu/plate, and Zero CFU/plate. All the results were under the specification limit. Environmental microbial counts are taken during the finished product preparation to ensure that no contamination has occurred and that microbial contamination control at the production area is within the specification limit. The production area was classified as class 1000, microbial count according to these classes must not more than 10 CFU/plate, the microbial count of this area it will depend on filtration of air that supply to these rooms [24,25].

Table 4. Microbial count at production area Class (1000) as a settled plate

Points	TAMC (TSA) CFU/plate	TYMC(SDA) CFU/plate	Standard. class 1000 (B) (MHRA,2017&WHO, 2011)
1	2	Zero	10
2	5	3	10
3	4	5	10
4	8	6	10
5	1	1	10
6	3	Zero	10

CONCLUSION

The present study declared that five non-sterile pharmaceutical raw materials were collected from Egyptian pharmaceutical company. Evaluation of different methods to determine microbiological quality control was obtained. This method is applied according to pharmacopeia. The results of five non-sterile pharmaceutical raw materials were recorded as high quality and limited in the total microbial count. After applying this method, we ensure enter these pharmaceutical raw materials into the production area. Also, evaluation of environmental conditions in the laboratory and production area was done using methods for microbiological quality control according to pharmacopeia. Results recorded acceptable total microbial count in laboratory and production area, so these environments are affordable for production of drugs according to WHO standards.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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